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Temperature manipulating peptide self-assembly in water nanofilm*

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Peptide GAV-9 is derived from 3 different disease related proteins. The self-assembly of GAV-9 in a water nanofilm attracted much attention recently. We studied how the temperature factor influenced the peptide self-assembly in a water nanofilm and found interesting phenomena: 1) the higher the temperature, the faster the nanofilaments grow; 2) the GAV-9 peptide formed double monolayers in a water nanofilm at 60 °C, which further supports the hypothesis that the water nanofilm could change the hydrophobicity of mica. We believe these results can help not only the microcontact printing of amyloid peptides, but also a better understanding on how temperature controls the properties of water nanofilm.

Keywords: Peptide self-assembly, Water nanofilm, Microcontact printing, Nanofilament, Hydrohpobicity

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I. INTRODUCTION

Amyloid diseases, such as Parkinson disease, Alzheimer's disease, Creutzfeldt-Jakob disease, Type II Diabetes, often refer to insoluble misfolded protein forming fibrils and their depositing inside cells, which eventually leads to cell death and related function loss [1–4]. By studying the fibrillization process of amyloid proteins and their conserved peptide sequences, it is believed that the pathogenic mechanism of these kind of diseases could be understood better [5–9].

We previously synthesized a short peptide termed GAV-9 (NH_2-VGGAVVAGV-$CONH_2$) [10] by comparing the core/conserved sequences of three amyloid proteins: α-synuclein [11–18], A-β [19–24], and prion proteins [25–28]. By using in situ liquid atomic force microscopy (AFM) imaging, we revealed the different self-assembling manners of GAV-9 in a bulk solution with the help of inorganic templates. On a hydrophilic mica surface, GAV-9 adopted "standing up in a line" to grow nanofilaments; on a hydrophobic highly ordered pyrolytic graphite (HOPG) surface, they preferred to lie down for self-assembling into nanofilaments. These results in a certain sense could reveal how interfacial hydrophobicity directs the different amyloidosis processes *in vivo*, because the mica and HOPG were believed to be the good mimics for biomembranes and the hydrophobic region of proteins [29–31].

In contrast to bulk water, water confined to a nanometer scale exhibits unique properties, thus has recently attracted much attention from scientists [32–35]. For cells, it is believed that most of intracellular water might be in a nanofilm state because of a crowded intracellular environment [36]. To figure out whether this unique water nanofilm influences GAV-9 peptide self-assembling, we fabricated a depth-controllable water nanofilm system and put GAV-9 molecules in it by employing μCP technique [37, 38]. The results show that GAV-9 adopted a "lying down" manner to grow filaments on a hydrophilic (mica) substrate at a relatively slow rate [39]. The "lying down" fibrillization manner resembles the behaviour of GAV-9 assembling on a HOPG surface. However, difference exists compared this "lying down" manner in nanowater film on a hydrophilic mica to that in bulk water on a hydrophobic (HOPG) surface. In a water nanofilm, the fibrillization rate is much lower and there are no multilayers formed; even with excessive GAV-9 molecules [37, 38]. Despite the debate of whether the water nanofilm could really change the hydrophobicity of mica surface, temperature is another common factor that has not been studied so far. Although a number of papers have already studied how temperature influences a molecule's self-assembly in a bulk solution, for water nanofilm this should be the first time.

In this paper, by systemically studying the self-assembly of GAV-9 in a water nanofilm under the same humidity and different temperature, we have at least answered three questions: i) Why the amyloid peptides do not form fibrils when they are lyophilized from a solution. ii) How to obtain consistent results with μCP amyloid peptides. iii) How multilayered GAV-9 films can be formed in the water nanofilm since the water nanofilm can mimic the HOPG surface.

II. EXPERIMENTAL SECTION

A. Peptide sample preparation

Peptide GAV-9 lyophilized powder was purchased from Beijing Ze Xi Yuan Biological Technology Co. Ltd. The final purity of the peptide is more than 95%. Before use, GAV-9 was freshly dissolved in Milli-Q water to a final concentration of 3.2 mM.

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B. Fabrication of stamps and μCP of peptides

We referred to the previous protocols [37] with minor modifications. Briefly, polydimethylsiloxane (PDMS) stamps were fabricated by pouring a 10 : 1 (w:w) mixture of Sylgard 184 elastomer/curing agent (Dow Corning) over a master with 1.6 μm pitch on its surface and heating at 70 °C for 12 h after degassing. The μCP processing of peptides followed the steps in Fig 1. The transfer processes were conducted at 25 °C and relative humidity (*RH*) of 40–50%.

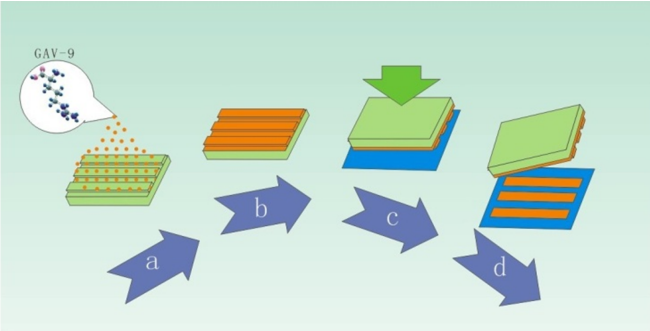

Fig. 1. (Color online) Scheme for the μCP process. a) Drop 20 μL GAV-9 solution (3.2 mM) on the surface of PDMS and stay for 5 min; b) Remove the solution and wait for 5 min until the PDMS surface was dried; c) Print the peptide side onto the mica surface and keep 5 min for high efficient transferring; d) Stripped patterns of peptide monomers were printed on the mica surface after removing the PDMS master.

C. Incubation of GAV-9 in water nanofilm system

This also referred to the previous protocol [37] with minor modifications. Briefly, the thickness of the adsorbed water layer has been found as a function of the relative humidity and temperature [40]. Thus, we can get water nanofilms with varied thicknesses and structures by controlling the environment temperature and relative humidity. In our experiment, freshly cleaved mica with the strips of GAV-9 were incubated in a climatic chamber (SDH-01N, Shanghai Jianheng Instrument Company) at strictly controlled temperatures (±0.1 °C) and *RH* (±5%).

D. Atomic force microscopy

A commercial AFM instrument (Nanoscope IIId, Bruker) equipped with a J-scanner (125 μm × 125 μm) was employed. The tapping mode in air was performed to observe the peptide strips on mica and their self-assembling results after different incubations. Commercial silicon cantilevers with normal spring constants of ∼48 N/m and a resonant frequency of ∼300 kHz (Tap300 AI-G, Budget Sensors) were used. All images were captured with a scan rate at 1–2 Hz and 512 samples × 512 lines resolution.

III. RESULTS AND DISCUSSIONS

In all of the experiments, GAV-9 molecules were first printed on the mica surface in a stripped pattern by μCP (Fig. 1) to act as the monomer source for the self-assembling process.

In order to conduct reliable comparison experiments, the state of the monomer source must be comparable for both the strip's height and homogeneity. However, for most of the operators, both of these two parameters could not be well controlled from time to time even under the same operation condition (25 °C and *RH* 45%, Fig. 2), which was time consuming.

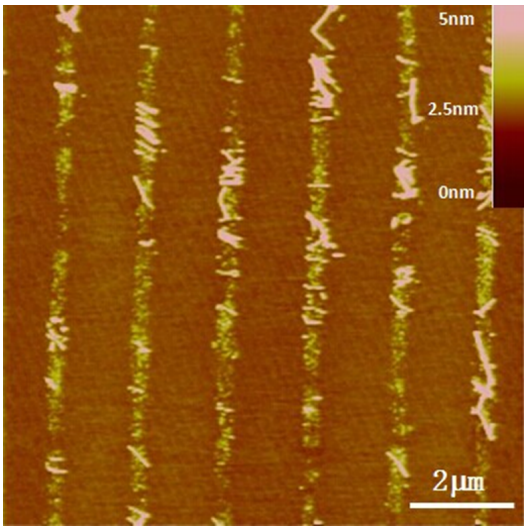

Fig. 2. (Color online) The GAV-9 solution containing fibrils was printed on a mica surface by μCP. GAV-9 already aggregated into fibrils with a diameter of 1–1.5 nm in solution before μCP, which was printed on mica. This image was obtained just after μCP.

From the μCP results checked by AFM (Fig. 2), we found that there were lots of fibril-like aggregates located on the peptide strips with a diameter about 1–1.5 nm, which we think are the main culprit in disrupting the μCP process because these aggregates prevent the well contact between mica and PDMS master. The fibril-like aggregates formed in the solution can be proved by hydrodynamic size monitoring by the dynamic light scattering (DLS) method (Fig. 3). Due to the hydrophobic property of GAV-9, they are prone to aggregate in the water solution with time. Since peptide self-assembling is an entropy driven process, the aggregation/fribrilization is also temperature dependent. Fig. 3 shows that even stored in a 4 °C refrigerator, GAV-9 can aggregate in days.

To answer why the lyophilized peptide powder can still keep its monomer state (Fig. 4), we must know that during lyophilisation, the peptide solution was first cooled below minus 50 °C within hours and then the vacuum cause a relatively slow sublimation until the frozen state became completly dried powder. So we speculate that such low temperature (< -50 °C) minimize the self-assembling rate since the solid water crystal cells prevent the peptide molecules from

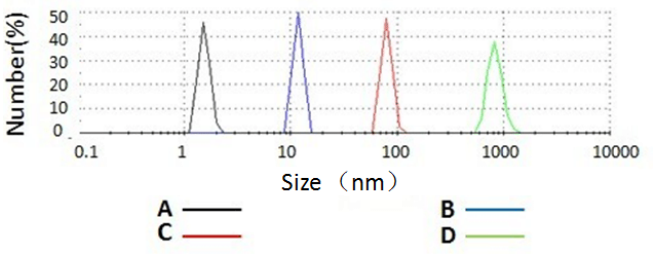

Fig. 3. (Color online) The hydrodynamic size of the peptide solution at different storing time. The hydrodynamic diameters of the GAV-9 water solution (3.2 mM) stored at 4 °C for different time measured by DLS (Malvern Instruments Ltd., ZS90). A) Freshly prepared solutions with an average diameter of 1.5 nm; B) Stored for a week showed an average particle size of 11.7 nm; C) Stored for a month with an average diameter of 78.8 nm; D) Stored for four months with an average diameter of 841.3 nm. All the diameter values were averaged by number.

colliding with one another. The followed sublimations securely and tenderly keep their monomer state by stealing the water molecules away, which might further indicate how important water is for hydrophobic interaction.

With the above knowledge, we know there is only one way to get reproducible μCP experiments. We have to use freshly prepared GAV-9 solution for each μCP operation and the left solution must be lyophilized as soon as possible if one still want to reproduce the results. Although it seems complicated and time consuming, we have not found other effective methods so far. Moreover, once the aggregates form, it is very difficult to re-dissolve them into monomer states. We want to emphasize the significance of keeping the GAV-9 peptide as a monomer in the solution. It is a critical requirement for reproducible μCP results. In Fig. 4, we got repeatable and homogenous GAV-9 strips with an average height of ∼5 nm, a width of 0.35 μm, and an interval of 1.2 μm on the mica surface.

To figure out how temperature influences the self-assembling process of GAV-9 in a water nanofilm, we utilized AFM as a powerful analysis tool for this study by varying temperature and keeping *RH* identical (70%). From the AFM results (Fig. 5), we can clearly see at low temperature (5 °C), the GAV-9 molecules can diffuse quickly but cannot form observable nanofilaments until 48 h. In contrast, at 37 °C, GAV-9 can form observable nanofilaments even after 12 h of incubation, and further grow into many apparent nanofilaments until 48 h incubation. At 60 °C, to our surprise, we found double nanofilament layers in nanowater film (Fig. 5(l)), which had been found on the hydrophobic HOPG surface in bulk water previously [39]. The reason we termed it double layers can be supported by the cross-section analysis results (Fig. 6), in which the upper nanofilaments' heights are ∼ 1.9 nm, whereas the bottom ones' are ∼ 0.9 nm.

From our previous *in situ* AFM results [39], GAV-9 can form multiple layers on the HOPG surface in bulk water very readily and quickly. We attribute this phenomenon to higher temperature, which increases the whole self-

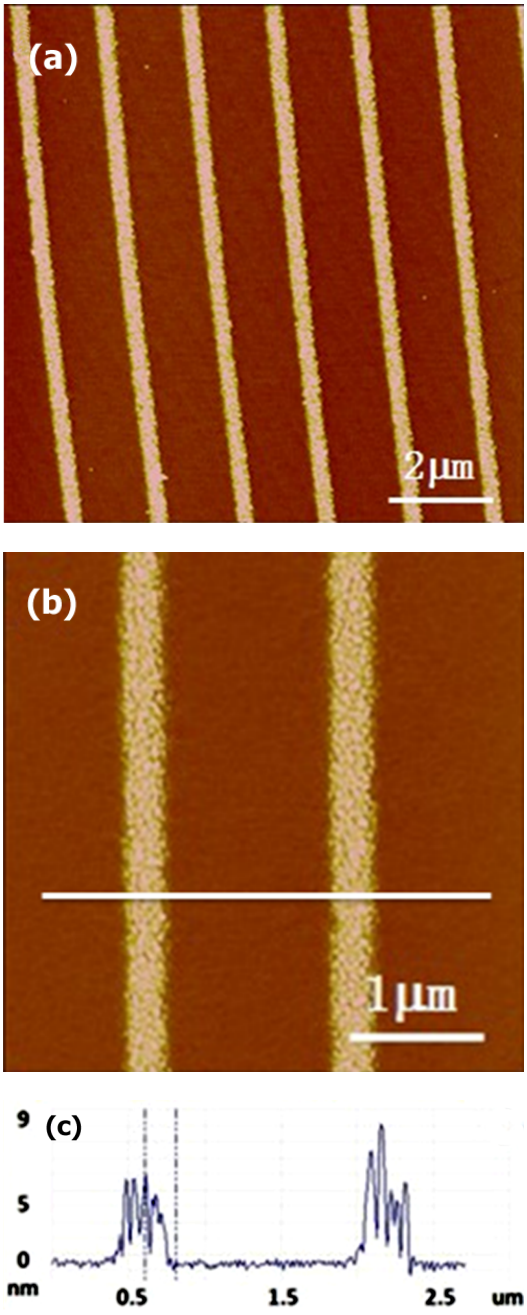

Fig. 4. (Color online) Stripped patterns of GAV-9 on Mica. (a) AFM height image of stripped GAV-9 patterns on mica surface, resolution: 256 samples × 256 lines, (b) An amplified AFM height image of GAV-9 strips from (a) to show there are no fibrils before the self-assembling process in a water nanofilm, resolution: 512 samples × 512 lines; (c) The cross-section analysis of GAV-9 strips' height from (b).

assembling system's entropy, so that peptide molecules can diffuse much faster. Though the self-assembling speed rose, the nanofilaments in both double layers still kept three-fold symmetry, which reveals the "epitaxial" relationship between the peptide and the underlying mica lattice. This is consistent with the previous findings [37]. Ye *et al*. had previously re-

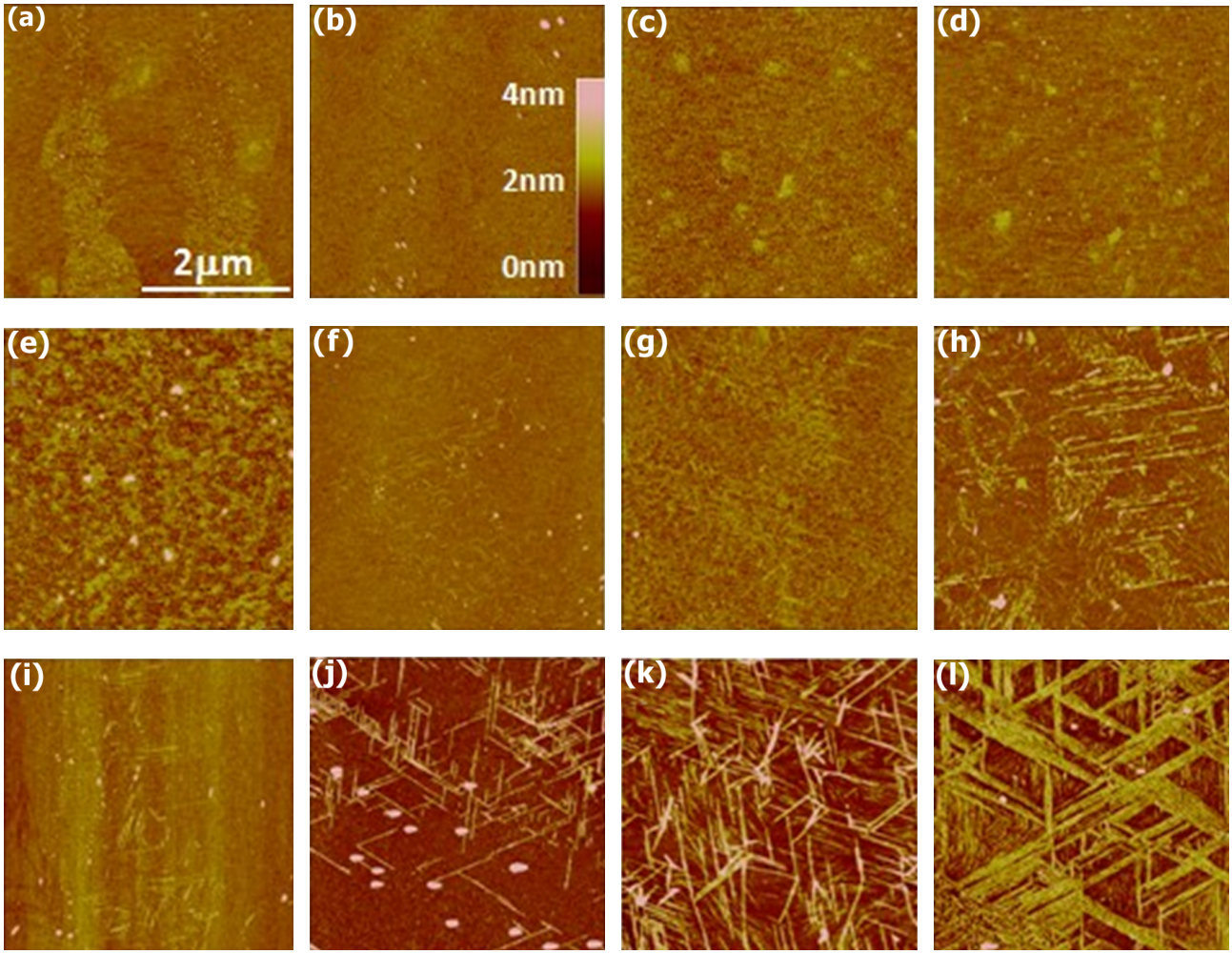

Fig. 5. (Color online) Time-series AFM snapshots of the GAV-9 diffusing process in water nanofilm at different temperatures. a-d) at 5 °C; e-h) 37 °C; i-l) 60 °C; All the images were recorded at no more than 20% RH, with a resolution of 512 samples × 512 lines; and for each temperature series, from left to right the four images were incubated for 12, 24, 36 and 48 h, respectively.

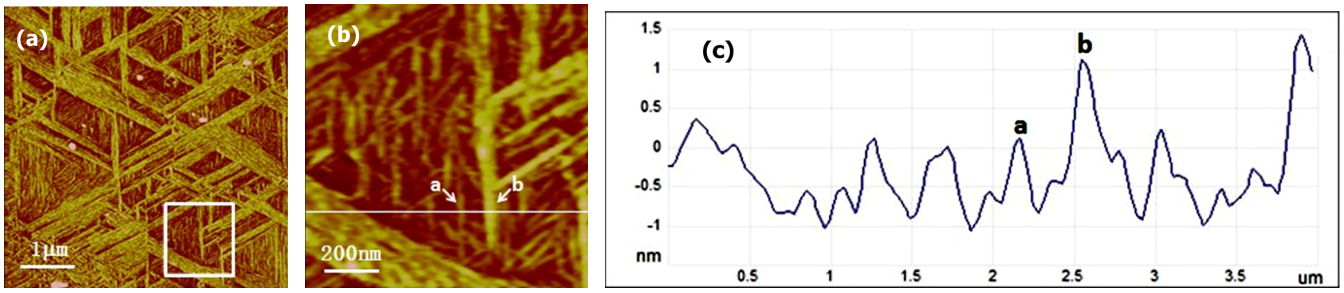

Fig. 6. (Color online) Analysis of the double layers of GAV-9 nanofilament film formed at 60 °C. (a) AFM height image of GAV-9 nanofilament double layers formed after 48 h incubation, resolution: 512 samples × 512 lines; (b) the magnified region of the marked rectangle in (a), arrows marked by **a** and **b** indicate the nanofilaments from bottom and upper film, respectively; (c) the cross-section analysis of the marked line in (b) and the letter **a** and **b** mark the height of nanofilaments corresponding to the position in (b) and **a** filament's height is ∼ 0.9 nm, **b** filament's height is ∼ 1.9 nm.

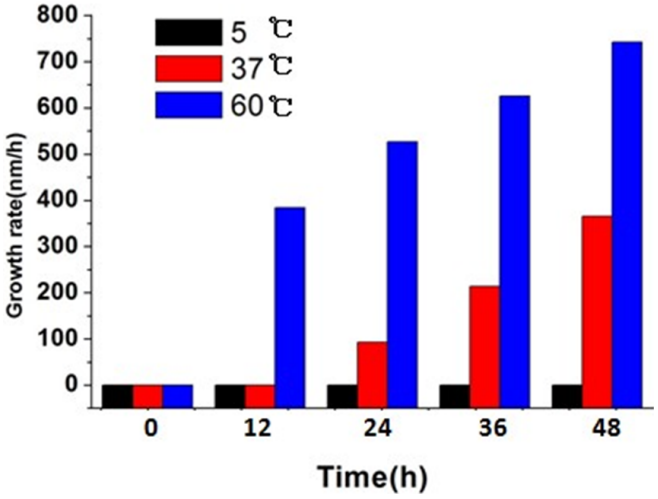

Fig. 7. (Color online) Nanofilament growth rate comparison of GAV-9 in water nanofilm at different temperatures. All the growth rate data was calculated by using nanofilament length divided by growth time in a 25 μm^2 AFM height image.

ported "double-layered" fibril structures under saturated RH at low temperatures [38]. From the figures in their paper, we can clearly see that the fibrils hadn't formed a real second layer. We would say the "double-heighted" fibrils stayed side-by-side. By comparing their incubation conditions with ours in this paper, it is clear that 100% RH at 25 °C was less potent than the 70% RH at 60 °C in terms of promoting peptide self-assembling in a water nanofilm.

To gain statistical knowledge of nanofilament growth influenced by temperature, we have calculated the nanofilment growth rate in a water nanofilm (Fig. 7). It is clearly shown that the higher the temperature is, the faster the nanofilaments grow, further indicating this is an entropy driven self-assembly process in which temperature plays a dominant role. By combining all of our previous results with the current results in this paper, we can figure out that both the thickness of the water nanofilm (since it is a function of RH at a fixed temperature [40]) and the temperature can manipulate the self-assembling behaviour of peptides in a water nanofilm. We can envision that more elegant nanostructures will be manmade by finely tuning the temperature and RH in the near future.

IV. CONCLUSION

By studying the effect of temperature on GAV-9 peptide self-assembly in a water nanofilm, we have figured out several unresolved questions. First, it is very important to keep the monomer state of the peptide solution to get reproducible results in the μCP process. To this end, one must freshly prepare the solution from the lyophilized peptide powder so that the lyophilisation won't cause the aggregation/fibrillization of the peptides. Second, the temperature is a paramount factor influencing peptide self-assembly in a water nanofilm both for the assembling rate and behaviour. At higher temperatures in a water nanofilm, the peptide self-assembles into 3-fold symmetric fibril patterns with a higher speed and even formed double-layered films, which exactly resembles the peptide assembling behaviour on the interface between bulk water and hydrophobic HOPG surfaces. We believe the above results and discussions will benefit the development of the μCP techniques and could be helpful to better understand the molecular self-assembly in water nanofilm.

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